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Improper regulation of the level and duration of activated erbB family growth factor receptors at the cell surface can lead to uncontrolled cell proliferation and transformation via over-stimulation of mitogenic signaling cascades. The large GTPase dynamin is a key regulator both of transport of receptors to the plasma membrane after receptor biosynthesis and down-regulation of receptors via receptor-mediated endocytosis (RME), during which it is involved in the scission of endocytic vesicles. Disruption of RME has been shown to render the epidermal growth factor receptor (erbB1) oncogenic (1), illustrating the importance of proper attenuation of signaling by down-regulation. This proposal addresses the mechanistic role of the pleckstrin homology (PH) domain in dynamin function, which may provide a pharmacologic target for modulating dynamin activity. The PH domain binds phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) at the plasma membrane (PM), but the role of this binding is not yet understood. The experiments detailed below address whether $PI(4,5)P_2$ binding is involved in targeting of dynamin to the PM, or whether phosphoinositide binding instead plays a more physical role in the scission of endocytic vesicles, and therefore receptor downregulation.

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## Introduction

Improper regulation of the level and duration of activated erbB family growth factor receptors at the cell surface can lead to uncontrolled cell proliferation and transformation via over-stimulation of mitogenic signaling cascades. The large GTPase dynamin is a key regulator both of transport of receptors to the plasma membrane after receptor biosynthesis and down-regulation of receptors via receptor-mediated endocytosis (RME), during which it is involved in the scission of endocytic vesicles. Disruption of RME has been shown to render the epidermal growth factor receptor (erbB1) oncogenic (1), illustrating the importance of proper attenuation of signaling by down-regulation. This proposal addresses the mechanistic role of the pleckstrin homology (PH) domain in dynamin function, which may provide a pharmacologic target for modulating dynamin activity. The PH domain binds phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) at the plasma membrane (PM), but the role of this binding is not yet understood. The experiments detailed below address whether $PI(4,5)P_2$ binding is involved in targeting of dynamin to the PM, or whether phosphoinositide binding instead plays a more physical role in the scission of endocytic vesicles, and therefore receptor down-regulation.

Here I summarize my work focused on characterizing the role of the dynamin PH domain in dynamin function, and the potential splice variant-specific roles of dynamin-2 in cellular trafficking. The necessary reagents for these experiments were successfully generated (Tasks 1, 2a and 3a). However, technical issues compromised the interpretation necessary to definitively assess the role of dynamin-2 splice variants in specific trafficking pathways (see Task 2 and Conclusions). Task 3, focused on characterizing the role of the dynamin PH domain, was very fruitful, and was expanded to include additional experiments than those proposed and completed from the original proposal.

## Body

This is the final status of progress compared with the original Statement of Work.

Task 1: Determine the subcellular localization of the four dynamin-2 splice variants.

a. Clone GFP-fusions of each dynamin-2 splice variant (months 1-5)
b. Isolate Clone 9 cell lines stably expressing each GFP-fusion (months 5-10)
c. Determine subcellular localization of each variant (months 11-15)

Task 1a: This task was completed in the second year. We successfully generated and/or obtained clones of all four dynamin splice variants as GFP-fusions, including (aa), (ab), (ba) and (bb).

Task 1b: This task was completed in the second and third years. We generated stable cell lines for GFP fusions of all dynamin-2 variants.

Task 1c: This task was completed in the second and third years. Consistent with the published literature, variant (ba) was found to be at the plasma membrane (2), variant (aa) was present at the TGN and the plasma membrane (3) and variant (ab) was found at only the plasma membrane (3).

Task 2: Demonstrate that the distinct subcellular localization demonstrated in Task 1 is mirrored by the role of each splice variant in cellular trafficking.

a. Clone each dynamin-2 splice variant of interest into tet-regulatable vector pUHD (months 15-18)
b. Determine the effect of dominant-negative mutants of relevant splice variants on receptor-mediated endocytosis (months 18-22)
c. Determine the effect of dominant-negative mutants of relevant splice variants on biosynthetic transport to the plasma membrane (months 23-28)
d. Perform *in vitro* TGN-budding assays with dynamin-2 variants (months 29-36)

Task 2a: Although subcloning of the dynamin-2 splice variants into the tet-regulatable vector was accomplished, the functional goal of this aim was delayed by the necessity to derive stable cell lines in a background with the tet-activator/repressor (tTA cell lines, in this case tTA HeLa cells), which was not initially outlined in the proposal, as constitutive expression of mutated dynamin-2 is toxic to cells. In addition, each cell line must maintain its tet-regulation, making isolation of these lines more difficult. Cell lines expressing Wt and mutant dynamin-2 (ba) were isolated and characterized first. The initial characterization of the dynamin-2 mutant expressing cell lines raised serious questions about the feasibility of assessing the effect of dominant-negative mutants (which must by definition be over-expressed to exert their effect) specifically on its particular cognate splice variant (eg (ba) on (ba) versus (aa), (ab), and (bb)). This technical issue seriously compromises our ability to carry out 2b,c, and d in a manner that will allow us to confidently assign a particular function to each splice variant. Alternative approaches using emerging technologies that utilize the reagents generated in Task 1 and Task 2a to effectively address these same objectives is discussed in the Conclusions section.

Task 2b, 2c and 2d: Initial assessment of the effect of the K44A and PH* mutations for the (ba) variant on receptor-mediated endocytosis raised concerns about the specificity of the over-expressed, mutated dynamin-2 constructs, as described above.

Task 3: Test the hypothesis that multivalent PH domain-mediated interactions are required for targeting of dynamin to clathrin-coated pits.

a. Purify recombinant wild-type and mutant dynamins (months 1-5)
b. Perform GTPase assays to test the effect of PH mutant dynamin on Wt dynamin (months 6-12)
c. Perform localization and internalization assays on the PH/T65A double mutant (months 18-30)

Task 3a: This task was completed in the first year. Both WT dynamin and dynamin with a mutated PH domain (PH*) were highly purified from baculovirus-infected Sf9 cells (Fig. 1), and additional dynamin mutant proteins utilized as controls below were cloned, expressed, and purified similarly.

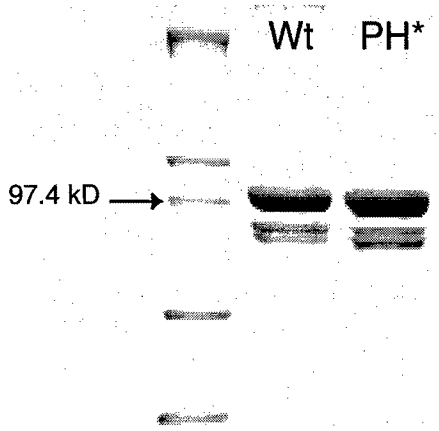


Figure 1. Purified dynamin Wt and PH* mutant (each approximately 100 kD) as analyzed by SDS-PAGE

Task 3b: This task was completed in the first year. First, the ability of vesicles containing the phosophoinositide $PIP_2$ to stimulate the GTPase activity of Wt and PH* dynamin was analyzed. As expected, the PH* mutant was essentially unable to undergo stimulation of GTPase by these vesicles (Fig.2A), despite the finding that induction of phosphoinositide-independent activation by low salt activated PH* as well as Wt. However, high concentrations of PH* dynamin still demonstrated significant GTPase activity at high lipid concentrations (Fig. 2B). This made this assay difficult to use to assess the potential dominant-negative activity of PH* dynamin on WT dynamin function. We therefore turned an assay that directly measures lipid binding (see below).

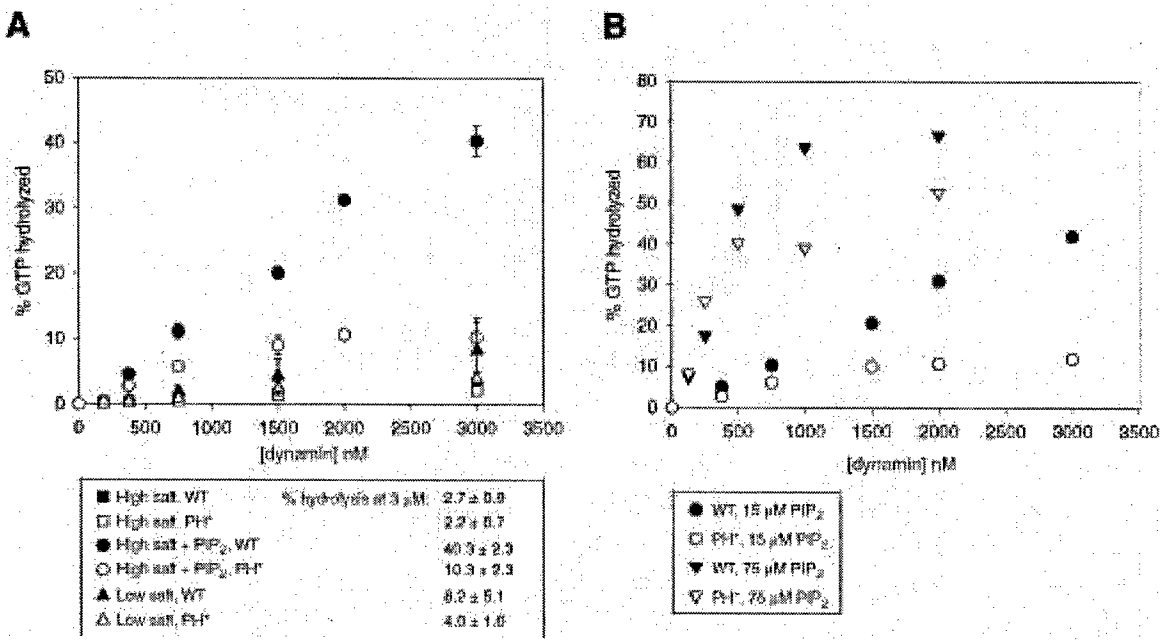


Figure 2. PH* dynamin is deficient in $PIP_2$-stimulated GTPase activity at limiting concentrations of $PIP_2$-containing vesicles. (A) Percent GTP hydrolysis at varying concentrations of WT or PH* dynamin after twenty minutes under conditions of high (137 mM) or low (67 mM) salt. $PIP_2$-containing vesicles (3 mole percent in PC) were added to 15 μM total lipid when indicated. Values in the legend refer to the percent GTP hydrolysis at 3 μM dynamin averaged from three experiments given with their standard deviations. Under these conditions the $PIP_2$-stimulated GTPase activity of PH* dynamin is approximately four-fold weaker than that of WT. (B) Comparison of $PIP_2$-stimulated GTPase activity of WT and PH* dynamin at varying $PIP_2$ concentrations (under high salt conditions) as indicated in the legend. Addition of excess vesicles reduces the difference seen between WT and PH* dynamin. Representative experiment from at least three repeats.

In order to look directly at the effect of PH* dynamin on WT dynamin binding to $PI(4,5)P_2$, we began using surface plasmon resonance (SPR). This is a more direct assay than the $PIP_2$-stimulated GTPase assay originally proposed. In addition we have included two control proteins for the effects of mutant dynamin on Wt dynamin binding. This includes K44A dynamin, which is deficient in nucleotide binding but should bind phosphoinositides similarly to Wt dynamin. An additional mutant, PH*/ΔCC, has the PH* mutation of the original PH mutant dynamin and a deletion of a critical coiled-coil sequence in the assembly domain that prevents multimerization. The addition of this coiled-coil deletion should revert the dominant-negative activity of the PH* mutant on Wt dynamin, as it cannot multimerize with the Wt protein.

The binding constants for each of these proteins to $PIP_2$ as evaluated by SPR were as predicted (Fig. 3A). Wt and K44A dynamin have similar binding affinities (around 200 nM), whereas both the PH* and PH*/ΔCC dynamin mutants are extremely deficient in $PIP_2$ binding with Kds in excess of 20 μM.

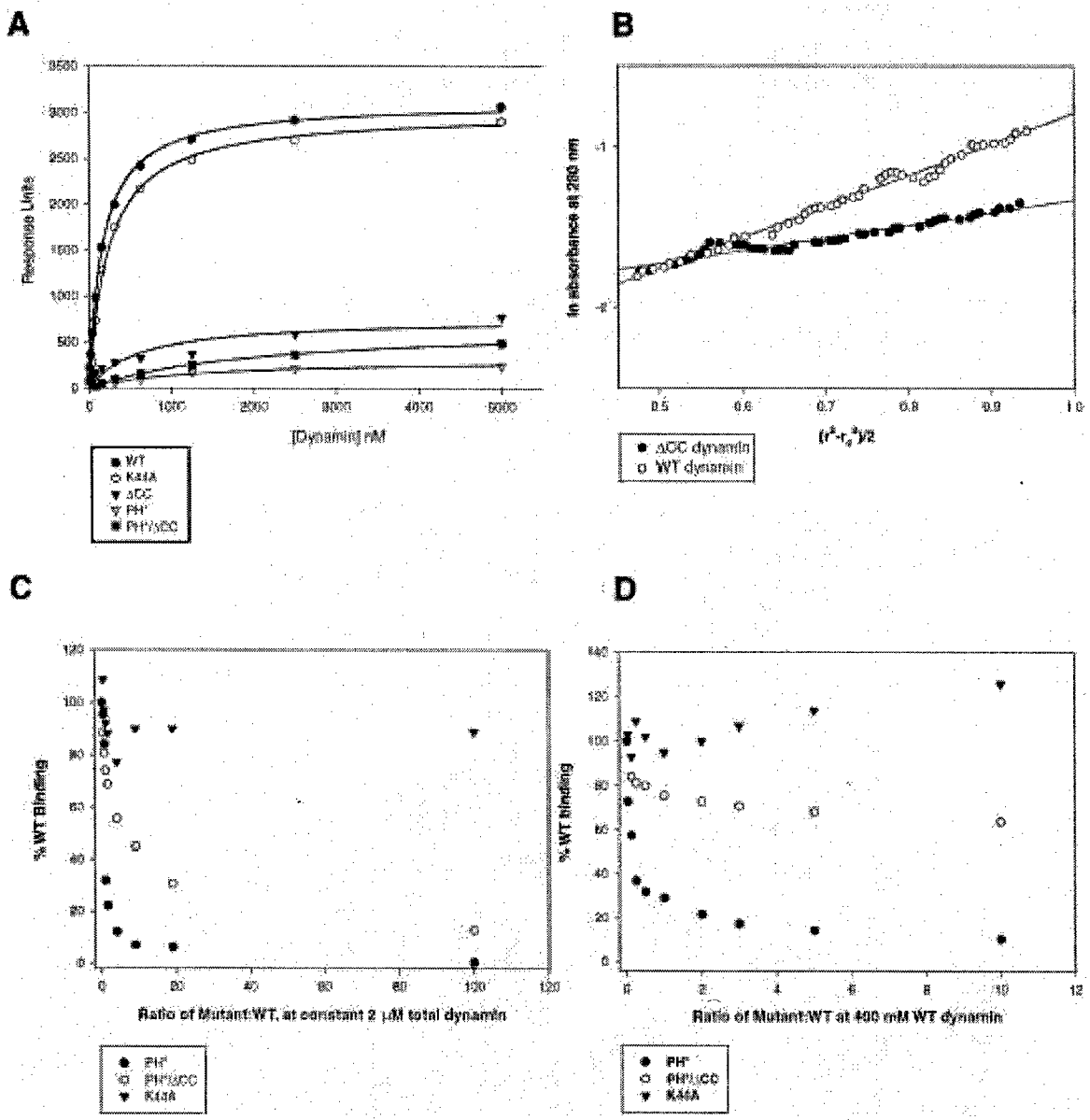


Figure 3. PH* dynamin can disrupt high affinity $PIP_2$ binding by WT dynamin *in vitro* in a dominant-negative fashion. (A) Binding of WT and mutant dynamins to a 10% $PIP_2$ /PC surface detected by SPR. WT and K44A (GTP-binding defective) dynamin bind strongly to this $PIP_2$ surface with $K_d$s of 205 ± 126 nM and 237 ± 35 nM, respectively. Disruption of oligomerization (ΔCC dynamin), introduction of the PH* mutation, or both result in a significant loss of binding (estimated $K_d$s > 20 μM). Binding curves and fits are representative of at least three experiments. (B) Deletion of the CC1 sequence in the GED domain (to give ΔCC dynamin) disrupts dynamin tetramerization as analyzed by sedimentation equilibrium ultracentrifugation, resulting in monomeric dynamin with an average molecular weight of 93.5 ± 6.5 kD compared to an average molecular weight of 347 ± 22 kD for WT dynamin. Representative distributions from a single speed and protein concentration are presented, and averaged molecular weights are calculated from at least six independent experiments. (C) WT and mutant dynamin were mixed at varying ratios (keeping the total dynamin concentration constant at 2 μM), and then their combined binding to $PIP_2$ was analyzed by SPR. Since K44A dynamin binds as WT, the total signal remains fairly constant at all ratios of mutant:WT protein. However, addition of PH* or PH*/ΔCC dynamin reduces the level of $PIP_2$ binding by the fixed 2 μM protein. Part of this effect results from the fact that the added mutants bind $PIP_2$ more weakly than WT. The strength of the effect for PH* dynamin is enhanced by a dominant-negative effect (see text and part D). Representative experiment from at least three repeats. (D) Increasing ratios of mutant dynamin were added to a constant concentration of WT dynamin (400 nM), so that the total [dynamin] in each mixture increase as mutant was added. The ability of each mixture to bind $PIP_2$ was then analyzed by SPR. PH*, but not PH*/ΔCC or K44A dynamin can disrupt binding of WT dynamin to the $PIP_2$ surface. Representative experiment from at least three repeats.

We next investigated the binding of mixtures of Wt and mutant dynamin proteins at a constant total protein concentration (2 μM). The results of these experiments are presented in Figure 3C. As Wt and K44A dynamin are equivalent in their binding to $PIP_2$, we expect that the binding should remain constant regardless of the ratio of Wt and mutant proteins. This was indeed found to be the case. For the PH*/ΔCC mutant, which cannot bind to $PIP_2$ efficiently, we expect the binding to slowly diminish as a larger proportion of the 2 μM protein is represented by the mutant. However, this is simply a dilution of Wt binding affinity as compared to the effect of PH* dynamin, which cannot bind $PIP_2$, but also inhibits binding of Wt dynamin. It is obvious from the effect of PH* versus the PH*/ΔCC that the ability of PH* to oligomerize with Wt dynamin is required to decrease the avidity of Wt dynamin for $PIP_2$, as represented by the highly potent effect of PH* dynamin.

This result was further investigated by mixing increasing ratios of mutant dynamin at a constant Wt dynamin concentration (400 nM). As seen in Figure 3D, these results are consistent with the conclusions above. Addition of K44A dynamin increases the total binding, as this mutant binds $PIP_2$ efficiently. There is only a slight effect of adding excess PH*/ΔCC dynamin, consistent with the inability of this mutant to oligomerize with Wt dynamin and affect its binding. Only PH* dynamin significantly disrupts Wt binding in a dose-dependent fashion. The efficacy of the PH* mutant dynamin is approximately analogous to the potency of this mutant at inhibiting RME *in vivo*, indicating that it is highly plausible that PH* dynamin exerts its effect by disrupting the avidity of $PIP_2$ binding by endogenous dynamin in the cell. This validates the hypothesis of Task 3, and demonstrates that PH* dynamin can act as a dominant-negative inhibitor of $PIP_2$ binding by WT dynamin.

Task 3c: This task is complete. Surprisingly, we found that the PH* mutation appears to affect neither the targeting nor inhibition of RME by the GTPase-deficient T65A mutant. Initial experiments demonstrated that high overexpression of T65A dynamin results in the formation of perinuclear aggregates (Fig. 4A). Expression of T65A dynamin and its derivatives were therefore controlled using the tet-system, as described in Task 2a. In Figure 4B, T65A dynamin is found to colocalize at trapped coated pits/tubules with GFP-clathrin. Deletion of the proline/arginine-rich domain responsible for initial dynamin targeting via the SH3 domain-containing protein amphiphysin disrupts this targeting (seen as a lack of colocalization of T65A/ΔPRD dynamin with clathrin). However, introduction of the PH* deletion (T65A/PH*) does not disrupt colocalization, indicating that phosphoinositide binding by the PH domain is not required for dynamin targeting. Therefore, the effect of PH* dynamin exhibited in Task 2 must occur at a stage of endocytosis after dynamin targeting.

Mutations known to disrupt targeting of dynamin (such as ΔPRD as described above) can revert the dominant-negative activity of a GTPase mutant when made in *cis*. When analyzed in a similar fashion, the PH* mutation is not able to revert the dominant-negative activity of T65A or K44A dynamin (Fig. 5). This suggests that the PH domain of dynamin is not involved in targeting of dynamin to nascent clathrin-coated vesicles, but may be involved more directly in the scission reaction promoted by dynamin. We have therefore gone on to address the effect of PH* dynamin at later stages of

endocytosis, which currently includes analysis of lipid tubulation and GTP-dependent constriction of lipid tubules as assessed by negative-stain electron microscopy. These results indicate that although PH* dynamin is capable of tubulating and constricting lipids as Wt dynamin, it does so with much reduced efficiency. Our further data suggests that WT, but not PH*, dynamin is able to cluster $PIP_2$ in a mixed lipid system. The data presented here, in conjunction with additional data, support a model where the PH domain of dynamin is necessary for lipid interactions as part of the scission mechanism of dynamin, rather than during the targeting of dynamin to its site of action.

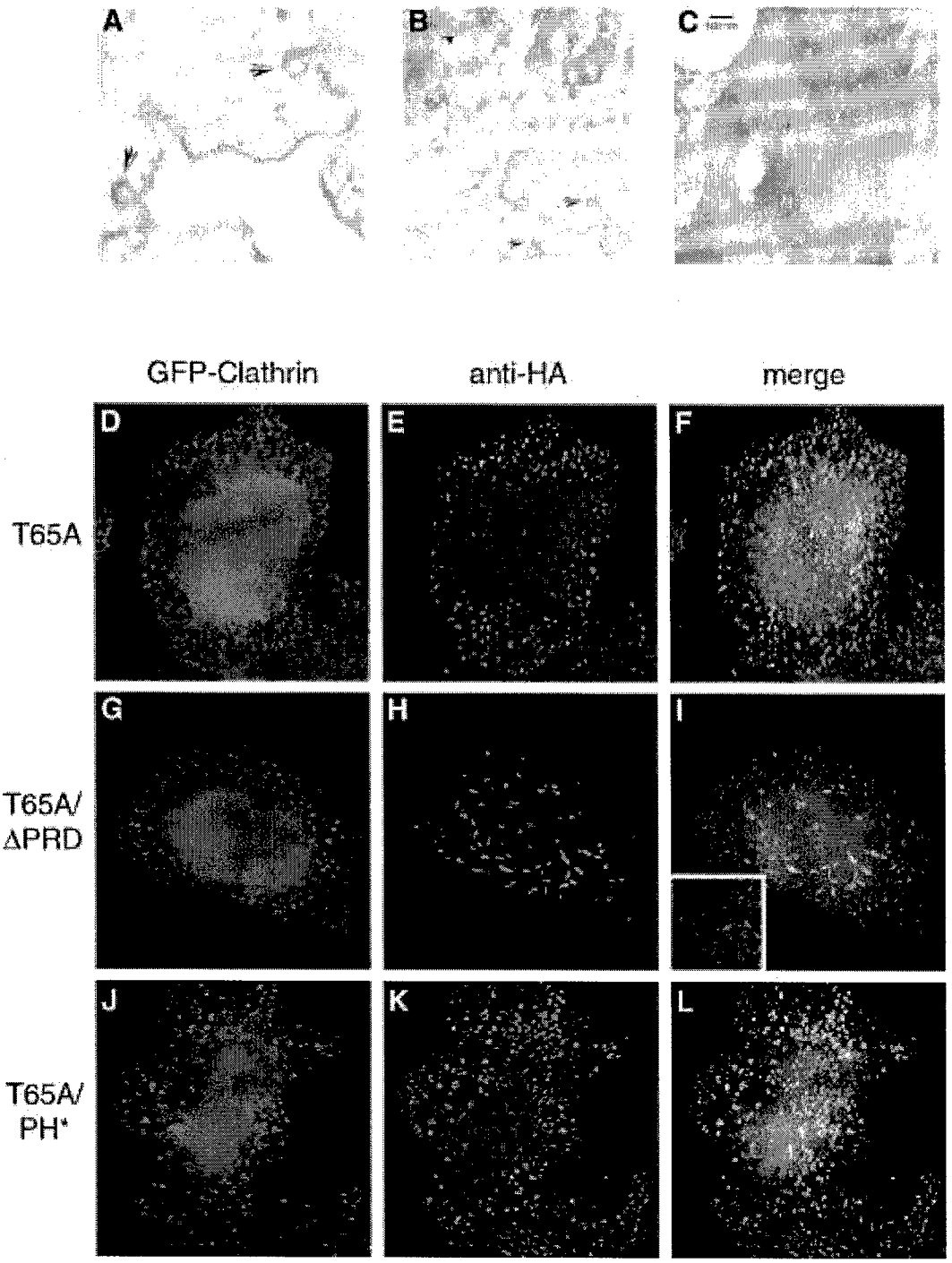


Figure 4. High-affinity $PIP_2$ binding by the PH domain is not required for localization of dynamin to endocytic structures. (A-C) Thin-section EM of HeLa cells expressing T65A dynamin. Several plasma membrane invaginations are seen (marked with arrows in A). However, a large bundle of tubules is present within the same cell when viewed at low magnification (black arrow in B). (C) The tubules do not appear to be membrane associated when viewed at high magnification. (D) Transfected T65A dynamin (detected with anti-HA) colocalizes with GFP-clathrin (E and F) at the plasma membrane by immunofluorescence in tTA HeLa cells when under moderate repression by inclusion of 100 ng/ml tetracycline in the growth media. (G-I) Disruption of T65A targeting by deletion of the PRD domain (to give T65A/ΔPRD) results in relocalization of the double mutant into perinuclear aggregates that fail to colocalize with clathrin. The inset in (I) demonstrates that the small amount of T65A/ΔPRD dynamin that is present at the plasma membrane is not coincident with clathrin. (J-L) Introduction of the PH* mutation (to give T65A/PH*) does not disrupt dynamin localization, and T65A/PH* colocalizes with clathrin as seen in (D-F). Representative images from at least ten fields of cells in each of three independent experiments.

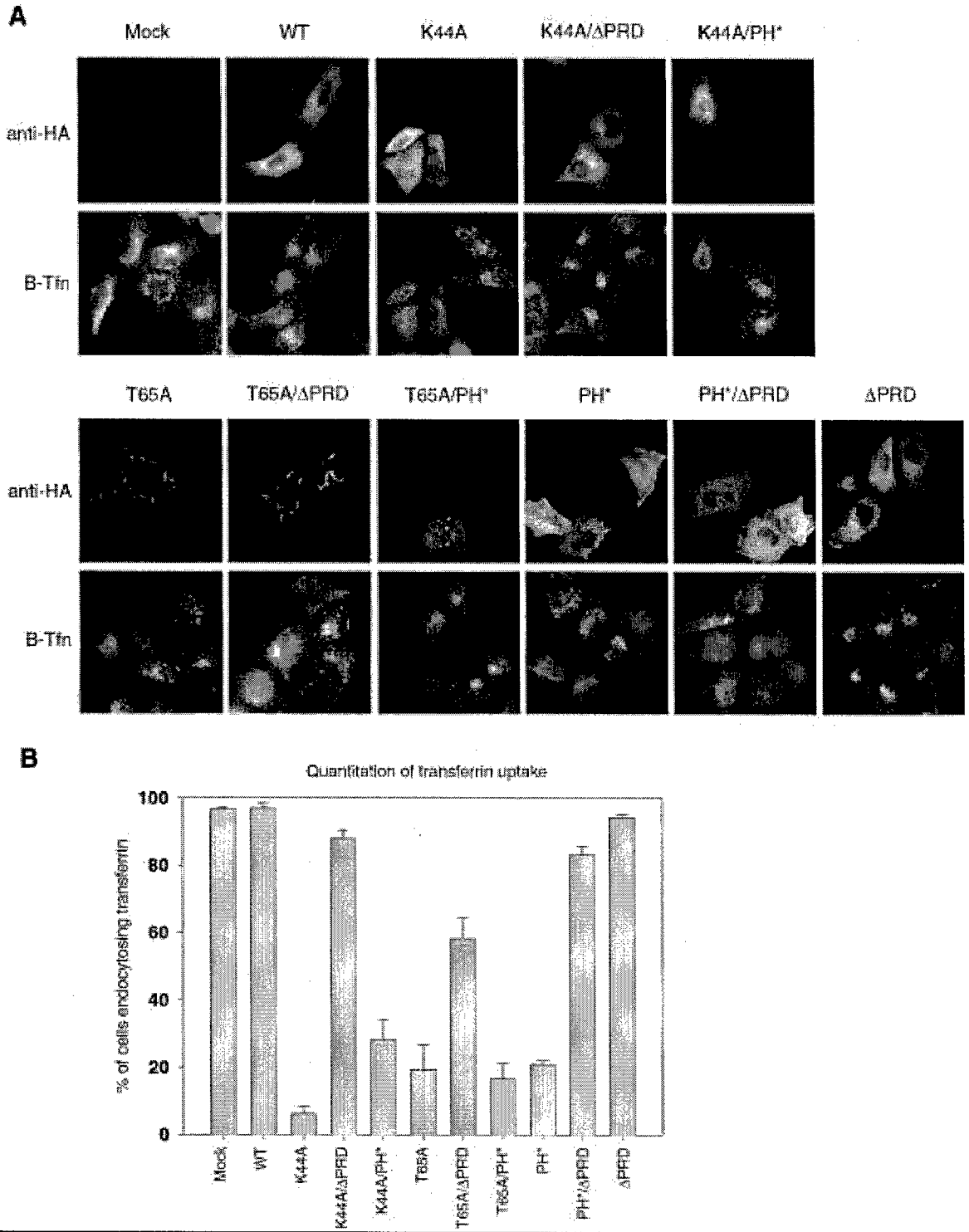


Figure 5. Mutation of domains that affect targeting can revert the dominant-negative effect of GTPase mutants, while mutations in effector domains are themselves dominant-negative inhibitors. (A) Cells transfected with HA-tagged dynamin (detected with anti-HA antibodies) and its mutants were tested for their ability to take up biotinylated transferrin (B-Tfn), detected with Texas Red conjugated streptavidin. Internalized B-Tfn appears as punctate cytoplasmic staining, whereas a failure to internalize B-Tfn results in a uniform cell surface distribution. Expression of the GTPase mutants K44A and T65A disrupts uptake of transferrin. Introduction of the ΔPRD deletion can revert these phenotypes, and cells expressing K44A/ΔPRD and T65A/ΔPRD are competent in transferrin endocytosis. Introduction of the PH* mutation is incapable of reverting the effect of these GTPase mutants, indicating that the PH domain is not responsible for targeting. (B) Quantitation of the percent of cells expressing the given dynamin construct that have internalized B-Tfn. Each experiment included at least 100 cells, and values are the averages of at least three experiments with their standard deviations.

**Key Research Accomplishments**

- Cloning of the dynamin-2 splice variants as GFP-fusions.
- Generation of stable cell lines for expressing GFP-fusions for the four splice variants.
- Cloning of WT and mutant GFP-fusions into the pUHD vector for tet-regulated expression
- Generation of tet-regulatable stable cell lines expressing dynamin mutants (partial)
- Purification of WT and mutated forms of dynamin from baculovirus-infected Sf9 cells
- Characterization of WT and PH* dynamin GTPase activity
- Validation of the hypothesis that the dominant-negative effect of PH-mutant dynamin on phosphoinositide binding by Wt dynamin, including key control experiments with other dynamin mutants
- Demonstration that introduction of the PH domain mutation does not prevent targeting of T65A dynamin to the necks forming clathrin-coated pits
- Demonstration that the PH* mutation is unable to revert the dominant-negative activity of dynamin GTPase mutants when made in *cis*, indicating a role for the PH domain in function rather than targeting

**Reportable Outcomes:**

Publications:

**King, M.C.**, Raposo, G, Lemmon, M.A. "Inhibition of nuclear import and cell-cycle progression by mutated forms of the dynamin-like GTPase MxB". P.N.A.S., USA. **101**, 8957-8962.

**King, M.C.**, Kelley A. Bethoney, Anthony Lee, Jenny E. Hinshaw, and MarkA. Lemmon. "The role of the dynamin PH domain in phosphoinositide clustering, not targeting, during dynamin function", *in preparation.*

Lee, A., **King, M.C.**, Lemmon, M.A. "The role of the dynamin PH domain in endocytosis". Poster, Gordon Research Conference: Protein Phosphorylation and Second Messengers, June, 2002.

**Conclusions**

We have successfully generated many reagents that have been and will continue to be useful in the study of dynamin. This includes GFP fusions of the dynamin-2 splice variants, which are widely expressed in all cell types. Although technical issues arose regarding our approach to assess the role of dynamin splice-variants in specific trafficking processes, these cell lines will be useful in the future for sophisticated live-cell imaging using photobleaching techniques. This type of approach will allow us to assess where and in what process individual splice variants act in the cellular context. Our investigations into the role of the dynamin PH domain have revealed a surprising role for

phosphoinositide binding in organizing lipids rather than targeting of dynamin to its site of action. Supported by additional experiments outside the scope of this proposal, this information will be instrumental in defining how modulating $PIP_2$ levels can affect dynamin-dependent trafficking, and therefore down-regulation of cell surface receptors. Currently we are confirming the localization studies performed here at the level of the electron microscope, and hope to submit the complete publication describing our findings regarding the role of the dynamin PH domain in RME within the next few months.

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